Biodegradation of Bisphenol A in a saline industrial wastewater using *Alcaligenes faecalis* strain BPAN5

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ABSTRACT

A large portion of petrochemical wastewaters are classified as high total dissolved solids, containing bisphenol A (BPA) which makes them resistant to conventional degradation processes. In this work, a halotolerant bacterial isolate, *Alcaligenes faecalis* strain BPAN5 was isolated and used for biodegradation of BPA. Effect of operational parameters including initial seed size (5–20 mL), nitrogen source, salinity (2%–3.5%) and BPA concentrations (10–60 mg/L) on BPA removal was investigated. Results showed that the addition of NH_4Cl in the culture medium led to the highest BPA biodegradation rate and bacterial growth. The most BPA removal of 100% and 96% were observed for the initial seed size of 15 mL and salinity of 2% for BPA concentrations of 10 and 20 mg/L, respectively. The main metabolites of BPA biodegradation were chlorobenzene, chlorothymol, hexadecanoic acid and 2,6-di-tert-butyl-4-phenylphenol. Also, the biodegradability of effluent reached 69% while the toxicity decreased to 7%. Findings of current work indicated the significant ability of *Alcaligenes faecalis* strain BPAN5 for biodegradation of BPA in a saline medium.

Keywords: Bisphenol A; Saline wastewater; *Alcaligenes faecalis* strain BPAN5; Biodegradation; Halotolerant bacteria

1. Introduction

The pollution of water by persistent organic pollutants and toxic contaminants has been a source of anxiety worldwide [1,2]. Fast-growing petrochemical industries and extensive use of petrochemical products led to environmental pollution during the last two decades. Petrochemical wastewater involves hazardous compounds such as halogenated hydrocarbons, aromatic hydrocarbons, and heavy metals which are harmful to human health and environment [3]. Moreover, a large portion of petrochemical wastewaters are classified as high total dissolved solids (TDS) effluents containing aromatic compounds, make them resistant to biodegradation [3]. Saline wastewater is defined as flow containing salt concentrations above 10 g/L [4,5]. In the petrochemical industry, polycarbonates and epoxy resin units use bisphenol A (BPA) as a raw material of the manufacture process [6]. Produced wastewater contains considerable amounts of salinity, which not only prevents bacterial metabolism but also decreases the efficiency of treatment processes [5]. Due to its estrogenic function on the mic and human stud, BPA is classified as one of the

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endocrine-disrupting chemicals (EDCs) [7]. EDCs are often referred to as external compounds that cause a change in functions of the endocrine system and interfere with production, secretion and natural hormones of metabolism [7]. BPA ($C_{15}H_{16}O_2$) belongs to the group of diphenylmethane derivatives [8]. BPA, as a monomer, is widely used in the production of polycarbonate plastics and epoxy resins, internal wall coverings of food storage containers, water bottles, and many other industrial products [9]. Increasing global demand for BPA has led to an increase in its production from 2.8 million tons in 2002 to about 5.5 million tons in 2015. It causes breast cancer, prostate cancer, reduced fertility, disorders of sexual development, and changes in the performance of the pituitary gland and thyroid [10]. Removal of BPA from contaminated wastewater should be considered during purification, following treatment standards [11]. Several methods have been studied for BPA removal from saline wastewater including biological methods and advanced oxidation processes [3,8]. Isolation and identification of salt-tolerant bacteria have been studied for the treatment of saline wastewater by different researchers. However, chemical methods have been successfully used for degradation of recalcitrant organics, but some drawbacks such as high energy consumption, chemical consumption, and secondary pollution due to the production of chemical residuals have been observed [5]. On the contrary, biological processes have simple operation, are efficient, cost-effective with less chemical demand [5,12,13]. But high salinity limits the biodegradation due to plasmolyzation biomass and disturbance of bacterial metabolism [5,14]. The required salt for microbial growth can be classified into different categories: (1) Non-halophiles (<0.2 M NaCl); (2) halotolerant (non-halophiles tolerating high salt concentrations); (3) slight halophiles (0.2–0.5 M NaCl); (4) moderate halophiles (0.5–2.5 M NaCl); (5) extreme halophiles (2.5–5.5 M NaCl) [15]. The halophilic bacteria have specific metabolic pathways via a high ability for the decomposition of a wide range of contaminants [5]. Based on the literature review, there are few studies on biodegradation of BPA in real saline wastewater and this is the first work introducing *Alcaligenes faecalis* strain BPAN5 as a halotolerant strain capable of BPA biodegradation. In this study, the ability of newly isolated halo-tolerant bacteria from saline wastewater for BPA degradation was studied.

2. Experimental

2.1. Materials

BPA ($C_{15}H_{16}O_2$), Acetonitrile (C_2H_3N) ≥ 95%, ethanol (C_2H_6O) 99%, NaCl, Sulphuric acid (H_2SO_4) (95%–97%), sodium hydroxide (NaOH) and all constituents of culture mediums were analytical grade and supplied from Merck, Germany. All stock solutions were prepared in deionized water.

2.2. Isolation of BPA-degrading bacteria

The isolation and enrichment of salt-tolerant bacteria capable of degrading BPA were performed based on procedure explain by Jorfi et al. [16]. First, a low TDS wastewater containing BPA originated from a petrochemical industry in the south of Iran was placed into a 250 mL flask, filled with 100 mL phosphate salt medium (PSM). The enrichment cultures included (g/L) K_2HPO_4, 6.3; KH_2PO_4, 1.8; NH_4Cl, 1; $MgSO_4·7H_2O$, 0.1; $CaCl_2·H_2O$, 0.1; $FeSO_4·7H_2O$, 0.1; $MnSO_4·H_2O$, 0.1 and 1 ml/L of trace elements solution, contained (g/L) H_3BO_3, 0.03; $ZnSO_4·7H_2O$, 0.01; $COCl_2·6H_2O$, 0.02; Na_2MoO_4, 0.006; $CuSO_4·2H_2O$, 0.001 [17]. The culture mediums were sterilized with an autoclave. Then, BPA was added as a carbon and energy source to enriched culture. Salinity was set to 2% using NaCl. The flasks were incubated at 31°C on a shaker incubator at 150 rpm for 7 d. The bacterial growth was carried out by measuring the absorbance at 600 nm ($OD_{600\ nm}$). The culture mediums were refreshed in 7 d intervals for six weeks to obtain BPA degrading isolate. To isolate pure BPA degrading strains, 1 mL of culture supernatant was diluted 10^{-4} times and distributed onto PSM agar plates containing BPA and incubated at 30°C for 48 h. Pure strains of morphologically separate colonies were isolated by duplicate streaking onto the BPA (50 mg/L) + agar PMS culture and selected based on growth capability.

2.3. Identification of bacteria

The identification of isolated bacteria was performed by Gram stain and morphological characteristics followed by the molecular method (Fig. 1). The genomic bacterial DNA was extracted using the Exgene Clinic SV kit (Gene All Biotechnology Co. Ltd, Korea). Bacterial 16S rDNA was amplified by universal previously-described primers fD1 and rD1 [17] following the method described in a previous study [5].

PCR product was purified and bidirectional sequencing of fragments was performed using an ABI 3730XL capillary sequencer (Bioneer Inc., South Korea). The sequence reads were edited and assembled using DNA sequence assembler v4 (2013). Sequences were compared against the Ez-Taxon (http://eztaxon-e.ezbiocloud.net) [18] and Gen Bank databases using the BLAST algorithm to determine the closest matching sequence identity. Evolutionary analysis was conducted in MEGA6 software. The tree was constructed using the neighbor joining algorithm with 1,000 bootstrap replicates [19].

2.4. Experimental procedure

All of the experiments were performed in 500 mL flasks. First, 100 mL of pre-prepared culture medium was transferred to considered flasks. Influence of operational parameters including nitrogen source (NH_4Cl, NH_4NO_3 and CH_4N_2O), initial seed size (5, 10, 15 and 20 mL), (salinity level 2%, 2.5%, 3% and 3.5%), BPA concentration (10, 20, 30, 40, 50 and 60 mg/L) and reaction time were investigated according to one factor at time experimental design. The experimental flasks were transferred on a shaker incubator at 150 rpm and 37°C.

2.5. Respirometry test

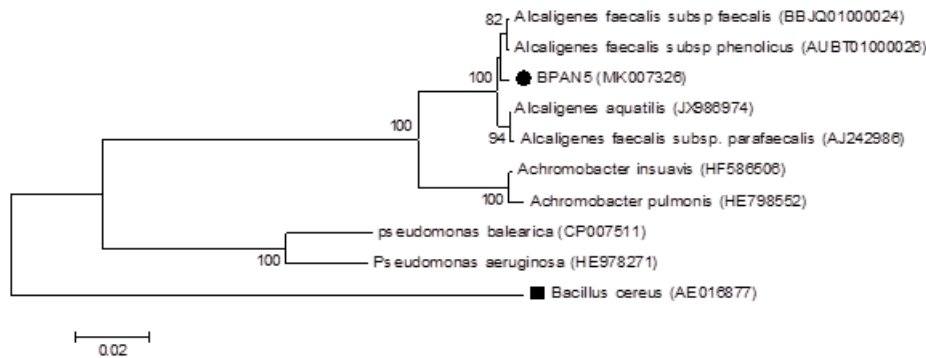A set of respirometry tests were carried out using a measurement of dissolved oxygen (DO) (DO meter, HACH)

Fig. 1. Phylogenetic tree of 16s rRNA sequences.

vs. time for an activated sludge sample obtained from a low-TDS industrial wastewater treatment plant (Mahshahr city, Iran) with mixed liquid suspended solids concentration of 3,000 mg/L. For each obtained effluent and BPA solution, 800 mL of low-TDS activated sludge was added to a flask (1,000 mL) and then 100 mL of the effluents was fed to it, while continuous aeration and shaking were conducted by an air pump and a shaker respectively. Oxygen uptake rate (OUR) was calculated by obtained slop from plotting DO vs. time. Biodegradability was achieved by comparing OUR values of the sample with a high biodegradable compound (acetic acid₁) based on Eq. (1). Toxicity was calculated according to Eq. (2) in which OUR value was measured for acetic acid being fed to a second time to the activated sludge that has been previously contacted with the sample (OUR$_{Aceticacid2}$) [20,21].

$$Biodegradablity(\%) = \frac{OUR_{Aceticacid1} - OUR_{sample}}{OURc_{Aceticacid1}} \qquad (1)[20]$$

$$Toxicity(\%) = \frac{OUR_{Aceticacid1} - OUR_{aceticacid2}}{OURc_{Aceticacid1}} \times 100 \qquad (2)[21]$$

2.6. Analytical methods

In order to analyze the BPA concentration, the first methanol was added to the culture and filtered to remove insoluble substances and cells. BPA was measured by a performance liquid chromatography (HPLC) (KNAUER, Germany) with a 2,500 ultraviolet (UV) detector system. To separate the solid and liquid phase, 50 mL of the sample was centrifuged at a speed of 4,000 rpm for 10 min. Samples were filtered with 0.45 μm fiberglass filters at Sigma-Aldrich (Whatman). In the next stage, 100 μL supernatant was injected into the HPLC with a column C_{18} at 30°C. The mobile phase included a mixture of acetonitrile/millipore water (50:50, v/v) at a flow rate of 1 mL/min at 214 nm [22]. To measure the adsorbed BPA in the sludge phase, the solid pellet was ultrasonicated using a 20 ml methanol-dichloromethane mixture. (1:1, v:v) twice. Then, samples evaporated at 30°C and the residue was dissolved with 2 ml of methanol [21]. Characteristics of the real wastewater sample including chemical oxygen demand (COD), BOD₅ (biological oxygen demand), TSS (total suspended solids), TDS, pH, and turbidity were

analyzed according to standard methods for the examination of water and wastewater [23]. All the experimental data are expressed in terms of arithmetic averages obtained from at least three replicates.

3. Results and discussion

3.1. BPA degrading isolate

According to the morphological observations (Fig. 1), four BPA degrading strains (3 g negative and 1 gram-positive) were isolated. The results indicated that *Alcaligenes faecalis* strain BPAN5 had the highest growth rate compared to the other isolated bacterial strains in the mineral salt medium containing BPA (50 mg/L, NaCl 2%). The strain *Alcaligenes faecalis* strain BPAN5 is a gram-negative, catalase and oxidase-positive, rod-shaped and motile bacterium. Based on 16sRNA sequencing, the organism was identified as *Alcaligenes faecalis* sp. BPAN5 (Genbank ID: MK007326)

The tree was constructed using the neighbor-joining method with MEGA 6.0. Bootstrap values over 70% (1,000 replications) were shown at each node. *Bacillus cereus* (AE016877) was used as the out-group.

3.2. BPA biodegradation

3.2.1. Effect of initial seed size

The initial size of the bacterial seed is an important parameter in the biodegradation of organic pollutants which affect the function of the microbial population. Results showed that the biodegradation of BPA was also affected by the initial volume of bacterial seed in the culture medium. In this regard, the pure strains of *Alcaligenes faecalis BPAN5* demonstrated the best performance in biodegradation of BPA in values of 15 and 20 mL. The removal efficiencies after 72 h for seed volumes of 5, 10, 15 and 20 mL were 20%, 24%, 32% and 33% respectively (Fig. 2a). As can be seen, the rate of BPA removal increased along with the concentration of seed size. In fact, the removal of BPA was instantly relevant to the density of live bacteria in the culture. According to the results, a slow startup may be related to the low primary bacterial seed [24–26]. The results showed that the seed size when is set to 5 mL has less effect on the BPA biodegradation.

When the bacterial concentration is too high, the base number of colonies is large and a shortage of nutrients
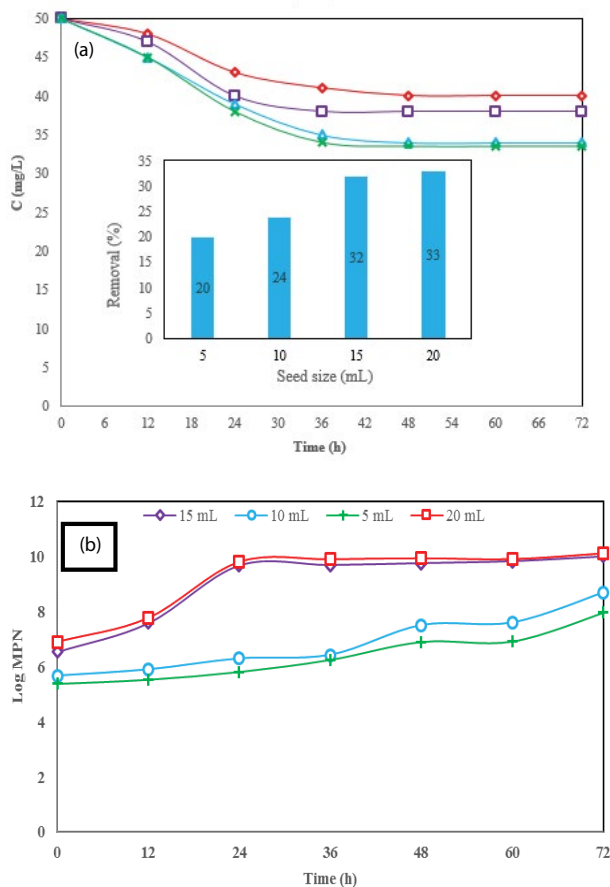
Fig. 2. Effect initial seed size on biodegradation of BPA, (a) removal efficiency and (a) variations of bacterial density (BPA 50 mg/L, salinity 2%, nitrogen source: NH_4NO_3).

affects the growth of bacterial strains [26]. Also, the results showed that when the highest inoculum density was used, no significant degradation enhancement was achieved and no further degradation of the BPA residues was observed after 1 d. This is due to the quick accumulation of antimicrobial metabolites in the primary stages of the growth and metabolism of strain sp. BPAN5 at a later phase, changing the degradation of BPA. Variations of bacterial density in Fig. 2b indicates that the higher removal values of BPA are supported by more bacterial population and more strength startup. Also, there was no significant difference between seed size of 15 and 20 mL and therefore, the seed size of 15 mL was selected for remaining experiments [27]. These results are supported by the literature [19].

3.2.2. Effect of nitrogen source

We studied the effect of various inorganic nitrogen sources (nitrate and ammonium) as well as organic kind (urea) as an important factor that could affect the growth and function of sp. BPAN5 cells. Various studies reported that the effects of organic and inorganic nitrogen vary greatly from one species or strain to another [28].

The effect of nitrogen source was investigated at BPA concentration of 50 mg/L, the salinity of 2% and seed size

of 15 mL (Fig. 3). It was seen that the growth of *Alcaligenes faecalis* strain BPAN5 and BPA biodegradation were affected by the different types of nitrogen sources. Among studied nitrogen sources, NH_4Cl yielded the most growth and BPA biodegradation. The removal efficiency of BPA via NH_4NO_3, NH_4Cl and CH_4N_2O, were 32%, 37%, and 18%, respectively. As shown in Fig. 3, the removal efficiency improved by increasing the reaction time for studied nitrogen sources from 6 to 24 h by the values of 18% to 30%, 6% to 34% and 2% to 18% for NH_4NO_3, CH_4N_2O, and NH_4Cl respectively. It can be said that the growth of sp. BPAN5 influenced by the type of nitrogen source obviously.

The nitrogen source of CH_4N_2O yielded low growth rates and BPA degradation since this is an organic source and bacterium strain needs to spend some energy on its reduction first. Therefore, there would be less energy for growth [29]. NH_4Cl is an inorganic source of nitrogen that enhances the ionic strength in water salinity. The result indicated that the absorption of urea by sp. BPAN5 leading to the excretion of the ammonium in culture. Cells with ammonia can adsorb the urea slowly in comparison to other sources of nitrogen [28]. Various studies reported that the effects of organic and inorganic nitrogen vary greatly from one species or strain to another. Nutrients that were adsorbed by sp. BPAN5 can affect cell growth and toxin generation. After senescence, dead cells are a source of substances that can be converted into dissolved mineral nutrients. Inorganic nitrogen in bacteria generated by living cells can be used to increase re-growth [28]. Consequently, if nitrogen is not available in the form of ammonia, excess energy is needed to convert the nitrogen source to ammonia. Therefore, NH_4Cl demonstrated the best results compared to other nitrogen sources.

3.2.3. Effect of salinity

NaCl is an essential element for the growth of the halo-tolerant microorganisms. Salt can regulate the osmotic pressure of the cell membrane and holding enzyme activity. Many studies have shown that salt concentration is a key parameter that has a significant effect on microbial growth [26,30]. To determine a threshold range for the action of isolated salt-tolerant strain, a series of experiments with different salinities were carried out [5]. The removal efficiency of BPA (BPA concentration of 50 mg/L, nitrogen source: NH_4Cl, seed size: 15 mL) at salinity levels of 2%, 2.5%, 3% and 3.5% was 37%, 38%, 37.6%, and 19% respectively (Fig. 4a). Other studies reported that higher salt concentrations prevented the activity of cells that were not adapted to salt, and cause of long lag phase, while the physiological salt concentration can increase microbial activity. Moreover, moderate salt concentration was required to motivate microbial growth [30]. Pollard et al. [31] showed that salt could affect the protein structure of cells and denature enzymes in the cell. Chloride ion prevents the enzyme activity by feedback prevention. When the salt concentration is higher than a desired value, the structure of the protein will be changed, it will affect the gene expression and cause non-reversible damage to the cells. As a result, the lag phase is raised and cell growth is affected [30].

About the above statement, in higher salt concentrations, the certain surface area of a bacterial cell increases due

to cellular contraction and therefore increases the carrying capacity of nutrients. Also, salinity affects the cell membrane hydrophobicity at a specific concentration. For these reasons, increasing the salt concentration from 2% to 3.5% caused a gradual decrease in the biodegradation rate [3,24]. Also, with enhancing salt concentration, surface tension increases and emulsion activity decreases. A significant decrease in bacteria density in the salinity level of 3.5% verifies the adverse effects of high salinity on *Alcaligenes faecalis* strain BPAN5 (Fig. 4b). The main mechanism of salt tolerance in bacteria is internal sequestration of high concentration of a balancing solute to equal the salt concentration found external to the cell. K^+ balancing mechanism and presence of acidic proteins with low proportions of non-polar amino acids help the salt tolerance ability in halo-tolerant strains [5].
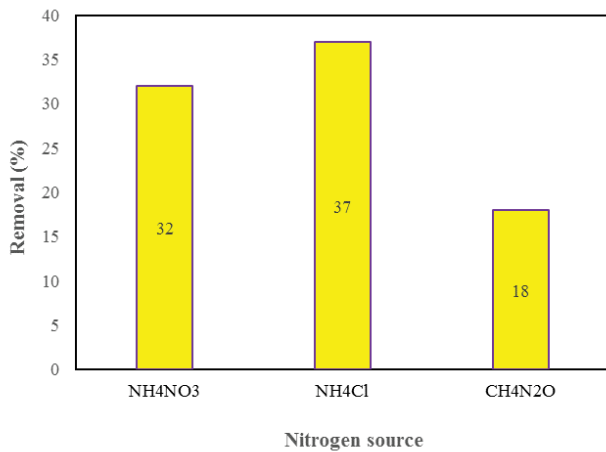
3.2.4. Effect of BPA concentration

Effect of initial BPA concentration at seed size of 15 mL, salinity of 2% and application of NH_4Cl as nitrogen source is indicated in Fig. 5. Results indicated that initial BPA concentration has an important effect on the cell growth and the rate of BPA degradation. According to Fig. 5, the maximum removal was observed at the BPA concentration of 10 mg/L. At higher BPA concentrations, cell growth was still high which in turn resulted in considerable biodegradation. Removal for initial BPA concentrations of 10, 20, 30, 40, 50 and 60 mg/L were 100%, 96.38%, 83.3%, 70%, 37% and 25%, respectively. High BPA concentration demonstrated an important inhibitory effect on microbial growth in concentrations above 50 mg/L since a short decline was observed in the bacterial growth curve [32,33]. This phenomenon is mostly called substrate inhabitation [34]. It was clear that BPA biodegradation by *Alcaligenes faecalis* strain BPAN5 was significantly dependent on the initial BPA concentration because of toxic impacts induced by the substrate [29]. The difference in growth rates and BPA tolerance characteristics of the strains could be due to their genetic modifications in different environments and different

Fig. 3. Effect of nitrogen type source on biodegradation of BPA (BPA 50 mg/L, salinity 2%, seed size 15 mL, reaction time 72 h).

Table 1
Intermediates of BPA biodegradation

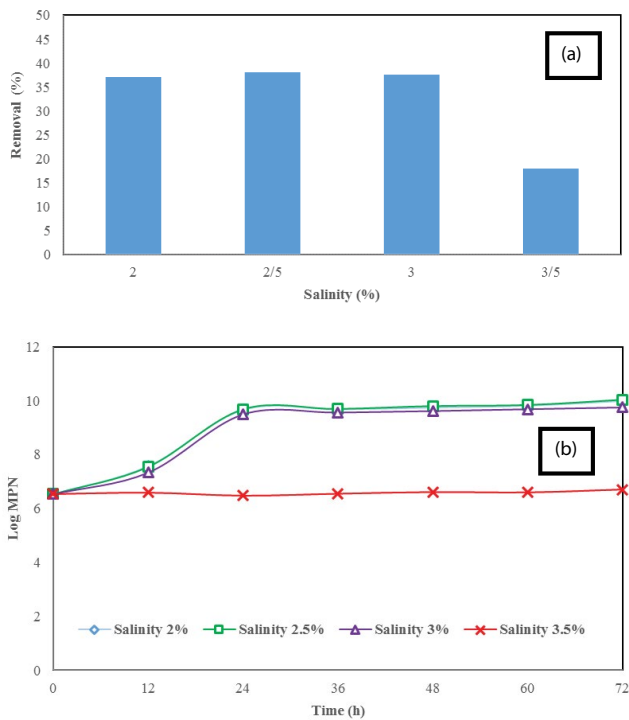| Intermediates	Area (%)
Chlorobenzene	14.8
Chlorothymol	0.11
Bisphenol	50.51
Hexadecanoic acid	0.3
2,6-di-tert-butyl-4-phenylphenol	0.28

Fig. 4. Effect salinity on biodegradation of BPA, (a) removal efficiency and (b) variations of bacterial density (BPA 50 mg/L, nitrogen source: NH_4Cl, seed size 15 mL).
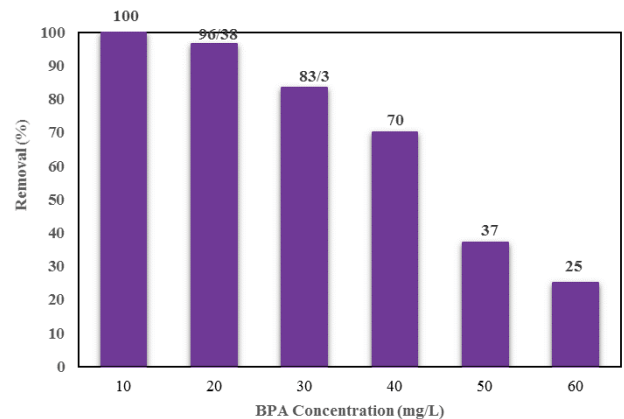
Fig. 5. Effect of BPA concentration on biodegradation efficiency using *Alcaligenes faecalis* strain BPAN5, (seed size: 15 mL, salinity 2%, nitrogen source: NH_4Cl).
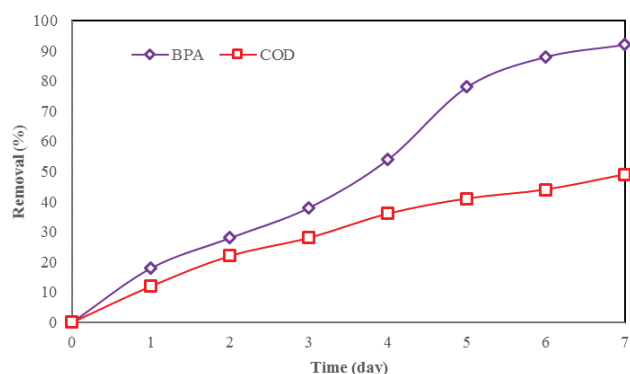
Fig. 6. Effect of reaction time on biodegradation and mineralization of BPA using strain sp. BPAN5, (seed size: 15 mL, salinity 2%, nitrogen source: NH$_4$Cl).

time of exposures. However, the degradation efficiency drastically falls for the initial BPA concentrations of above 50 mg/L [35].

3.3. Mineralization

Biodegradation would be preferred due to the potential for the complete mineralization of organics [36]. Mineralization of BPA was investigated using analysis of COD and BPA removal in selected conditions (salinity 2%, seed size 15 mL, NH$_4$Cl, BPA 50 mg/L and reaction time of 7 d). Experimental results showed that (Fig. 6) as regards of considerable BPA removal in 7 d (92%), COD removal reached only to 49% at the same time. A qualitative GC-MS analysis was used to survey the metabolites of BPA biodegradation with *Alcaligenes faecalis* strain BPAN5. The main intermediates of BPA biodegradation were Chlorobenzene, Chlorothymol, Hexadecanoic acid and, 2,6-di-tert-butyl-4-phenylphenol which were also seen in our analysis with some differences (Table 1) [37].

3.4. Biodegradability and toxicity assessment

The respirometry test was used for evaluation of biodegradability and toxicity of obtained effluents after performing a biodegradation study using *Alcaligenes faecalis* strain BPAN5 under reaction conditions of BPA 50 mg/L, nitrogen source NH$_4$Cl, salinity 2%, seed size 15 mL and contact time of 7 d. According to Fig. 7, a solution of 50 mg/L BPA had low biodegradability (11%) and high toxicity (74%) indicating the inability of activated sludge to deal with such recalcitrant solution. After biodegradation using *Alcaligenes faecalis* strain BPAN5, biodegradability was significantly improved and reached 69% while toxicity decreased to 7%. Based on these results, it can be deduced that *Alcaligenes faecalis* strain BPAN5 can oxidize BPA to non-toxic and biodegradable metabolites.

3.5. Real wastewater treatment

The mean concentrations of TDS, TSS, COD, BOD$_5$, and BPA of raw saline wastewater were 22, 740, 128, 470, 170 and 46 mg/L, respectively (Table 2). Due to the BOD$_5$/
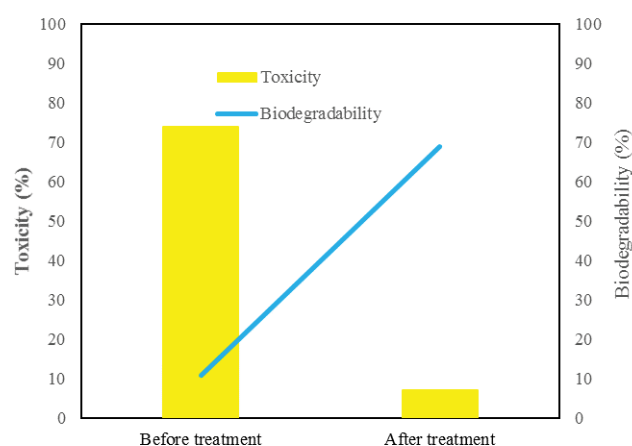

Fig. 7. Toxicity and biodegradability BPA solution (BPA 50 mg/L).

Table 2
Characteristics of a real petrochemical wastewater sample

Parameter	Initial concentration (mg/L)	Final concentration	Removal
		(mg/L)	(%)
COD	670	355	47.0149254
BPA	75	22	70.6666667

COD proportion of 0.38, this wastewater was characterized as a moderately biodegradable sample. There were two significant challenging properties of studied the wastewater: (i) low BOD$_5$/COD ratio and (ii) salinity values of more than 20,000 mg/L. The performance of isolated strain was studied for the treatment of a real petrochemical wastewater sample in selected conditions in 7 d. Results demonstrated a COD and BPA removal (%) of 43.6% and 70.6% respectively. Lower removal efficiency can be attributed to the presence of other target organics and the harsh environment of real wastewater.

4. Conclusion

A BPA containing petrochemical wastewater was subjected to biodegradation using isolated halotolerant strain, *Alcaligenes faecalis BPAN5*, after investigation of BPA biodegradation in a synthetic sample. The efficiency of PBA degradation increased with an increase in the concentration of microorganisms. Maximum removal was observed in BPA concentration of 10 mg/L, seed size of 15 mL and salinity: 2%. Degradation of PBA and intermediate compounds were determined using the GC-Mass technique and included Chlorobenzene, Chlorothymol, and Hexadecanoic acid and, 2,6-di-tert-butyl-4-phenylphenol.

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